RAFFINOSE SYNTHASE GENES AND THEIR USE

BACKGROUND OF THE INVENTION

FIELD OF INVENTION

The present invention relates to raffinose synthase genes and their use.

DISCLOSURE OF THE RELATED ART

Raffinose family oligosaccharides are derivatives of sucrose, which are represented by the general formula: o-α-D-galactopyranosyl-(1→6)$_n$-o-α-D-glucopyranosyl-(1→2)-β-D-fluctofuranoside, and they are called "raffinose" when n is 1, "stachyose" when n is 2, "verbascose" when n is 3, and "ajugose" when n is 4.

It has been known that raffinose family oligosaccharides have an effect of giving good conditions of enterobacterial flora, if present at an appropriate amount in food. Therefore, raffinose family oligosaccharides have already been used as a functional food material for addition to some kinds of food and utilized in the field of specific health food. On the other hand, raffinose family oligosaccharides are neither digested nor absorbed in mammals such as human, but are assimilated and decomposed by enterobacteria to generate gases and to cause meteorism and absorption disorder. Therefore, it has been desired to appropriately regulate the amount of raffinose family oligosaccharides in food and feed.

Raffinose family oligosaccharides are synthesized

by the raffinose family oligosaccharide biosynthesis system beginning with sucrose in many plants. This biosynthesis system normally involves a reaction for the sequential addition of galactosyl groups from galactinol through an α (1→6) bond to the hydroxyl group attached to the carbon atom at 6-position of the D-glucose residue in a sucrose molecule. Raffinose synthase is the enzyme concerned in the reaction for producing raffinose by allowing a D-galactosyl group derived from galactinol to form the α (1→6) bond with the hydroxyl group attached to the carbon atom at 6-position of the D-glucose residue in a sucrose molecule in the first step of this biosynthesis system. It has been suggested that this enzyme constitutes a rate-limiting step in the above synthesis system, and therefore this enzyme is quite important in the control of biosynthesis of raffinose family oligosaccharides.

Then, a method for controlling an expression level or activity of raffinose synthase in plants by utilizing a raffinose synthase gene is effective to control a biosynthesis system of raffinose family oligosaccharides in plants to increase or decrease the production of raffinose in plants. Thus, a raffinose synthase gene which can be used in such a method has been desired.

OBJECTS OF THE INVENTION

The main object of the present invention is to provide novel raffinose synthase genes from plants.

This object as well as other objects and advantage of the present invention will become apparent to those skilled in the art from the following description.

DETAILED DESCRIPTION OF THE INVENTION

The gene engineering techniques described below can be carried out, for example, according to methods described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press, ISBN 0-87969-309-6; "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X; "Current Protocols In Protein Science" (1995), John Wiley & Sons, Inc. ISBN 0-471-11184-8.

The genes of the present invention can be obtained from soybean, plants belonging to the families Chenopdiaceae such as beet, etc. and Cruciferae such as mustard, rapeseed, etc. Specific examples of the genes of the present invention include those comprising a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1, the nucleotide sequence represented by SEQ ID NO: 2, a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 3, the nucleotide sequence represented by SEQ ID NO: 4 or by the 236th to 2584th nucleotides in the nucleotide sequence represented by SEQ ID NO: 4, a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 5, the nucleotide sequence represented by SEQ ID NO: 6 or by the 134th to 2467th

nucleotides in the nucleotide sequence represented by SEQ ID NO: 6, a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 7, the nucleotide sequence represented by SEQ ID NO: 8 or by the 1st to 1719th nucleotides in the nucleotide sequence represented by SEQ ID NO: 8, and the like.

The genes of the present invention can be obtained, for example, by the following method.

That is, the genes of the present invention derived from soybean can be obtained, for example, by the following method.

For example, the gene can be obtained by a hybridization method using a nucleic acid having the nucleotide sequence represented by SEQ ID NO: 2 or its partial nucleotide sequence as a probe to detect a nucleic acid fragment which hybridizes to the probe in DNAs derived from soybean, followed by isolating the detected nucleic acid.

In this method, first, a nucleic acid to be used as the probe is prepared. As such a nucleic acid, for example, there is a nucleic acid composed of an oligonucleotide chemically synthesized by a conventional method on the basis of the nucleotide sequence of SEQ ID NO: 2. Specific example thereof includes a nucleic acid having the 800th to the 899th nucleotides in the nucleotide sequence represented by SEQ ID NO: 2.

Alternatively, the gene of the present invention derived from soybean can be obtained by the following method.

For example, tissue of soybean (*Glycine max*) is frozen

in liquid nitrogen and ground physically with a mortar or other means into finely divided tissue debris powder. From the tissue debris powder, RNA is extracted by a conventional method. A commercially available RNA extraction kit can be utilized in the extraction. RNA is recovered from thus-obtained RNA extract by ethanol precipitation. Poly-A tailed RNA is fractionated from thus-recovered RNA by a conventional method. A commercially available oligo-dT column can be utilized in this fractionation. cDNA is synthesized from the poly-A tailed RNA thus obtained by a conventional method. The synthesis can be carried out by using a commercially available cDNA synthesis kit. DNA is amplified by PCR using the above-obtained cDNA as the template and primers designed and synthesized on the basis of the nucleotide sequence of SEQ ID NO: 2. More specifically, as the primers, for example, there are primers 11 (SEQ ID NO: 9) and 12 (SEQ ID NO: 10) shown in List 1 hereinafter. When PCR is carried out by using these primers and as the template cDNA derived from soybean, the genes of the present invention derived from soybean, e.g., the "raffinose synthase gene having a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1" and the "raffinose synthase gene having the nucleotide sequence of SEQ ID No: 2" can be obtained.

The amplified DNA can be cloned according to a conventional method, for example, described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; or "Current Protocols In Molecular

Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X. Alternatively, cloning can be carried out, for example, by using a commercially available cloning kit such as TA cloning kit (Invitrogen) and a commercially available plasmid vector such as pBluescript II (Stratagene). The nucleotide sequence of the DNA clone can be determined by dideoxy terminating method such as that described by F. Sanger, S. Nicklen, A.R. Coulson, Proceedings of National Academy of Science U.S.A. (1977), 74, pp. 5463-5467. For example, preferably, a commercially available kit such as ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit manufactured by Perkin-Elmer can be used.

List 1

Primer 11 (SEQ ID NO: 9): ccaatctgat catgcttgtg ccgaa 25mer

Primer 12 (SEQ ID NO: 10): ggaacaaagt tatgcactat tatttaaggt 30mer

The genes of the present invention derived from a Chenopdiaceae plant such as beet can be obtained by the following method.

For example, tissue of a Chenopdiaceae plant such as beet (*Beta vulgaris*) is frozen in liquid nitrogen and ground physically with a mortar or other means into finely divided tissue debris powder. From this tissue debris powder, RNA is extracted by a conventional method. A commercially available RNA extraction kit can be utilized in the extraction. RNA is recovered from the thus-obtained RNA extract by ethanol

precipitation. From the recovered RNA, poly-A tailed RNA is fractionated by a conventional method. A commercially available oligo-dT column can be utilized in this fractionation. cDNA is synthesized from the poly-A tailed RNA thus obtained by a conventional method. The synthesis can be carried out by utilizing a commercially available cDNA synthesis kit. DNA is amplified by PCR using the above-obtained cDNA as the template and primers designed and chemically synthesized on the basis of the nucleotide sequence of SEQ ID NO: 4. More specifically, as the primers, for example, there are primers 21 (SEQ ID NO: 11) and 22 (SEQ ID NO: 12) shown in List 2 hereinafter. When PCR is carried out by using these primers and as the template cDNA derived from beat, the genes of the present invention derived from beet, e.g., the "raffinose synthase gene having a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 3," and the "raffinose synthase gene having a nucleotide sequence of SEQ ID No: 4" can be obtained. According to a particular purpose, the PCR primers can also be designed and synthesized on the basis of the nucleotide sequence of SEQ ID NO: 4. For example, in order to amplify the "raffinose synthase gene having the nucleotide sequence represented by the 236th to the 2584th nucleotides in the nucleotide sequence represented by SEQ ID NO: 4", preferably, oligonucleotides having the nucleotide sequences represented by primers 23 (SEQ ID NO: 13) and 24 (SEQ ID NO: 14) in List 2 below are synthesized and used as the primers.

The amplified DNA can be cloned according to a

conventional method, for example, described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; or "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X. Alternatively, cloning can be carried out by using a commercially available cloning kit such as TA cloning kit (Invitrogen) and a commercially available plasmid vector such as pBluescript II (Stratagene). The nucleotide sequence of the DNA clone can be determined, for example, by dideoxy terminating method such as that described by F. Sanger, S. Nicklen, A.R. Coulson, Proceedings of National Academy of Science U.S.A. (1977), 74, pp. 5463-5467. For example, preferably, a commercially available kit such as ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit manufactured by Perkin-Elmer can be used.

List 2

Primer 21 (SEQ ID NO: 11): ctaccaaatt ccacaactta aagttca 27mer

Primer 22 (SEQ ID NO: 12): ggaataataa gcttcacaca tactgtactc tc 32mer

Primer 23 (SEQ ID NO: 13): atggctccaa gctttagcaa ggaaaattcc 30mer

Primer 24 (SEQ ID NO: 14): tcaaaataag tactcaacag tggtaaaacc 30mer

The genes of the present invention derived from Cruciferae plants such as mustard (*Brassica juncea*) and rapeseed (*Brassica napus*) can be obtained by the following method.

For example, tissue of a Cruciferae plant such as mustard or rapeseed is frozen in liquid nitrogen and ground physically with a mortar or other means into finely divided tissue debris powder. From the tissue debris powder, RNA is extracted by a conventional method. A commercially available RNA extraction kit can be utilized in the extraction. The RNA is recovered from thus-obtained RNA extract by ethanol precipitation. Poly-A tailed RNA is fractionated from the RNA thus recovered by a conventional method. A commercially available oligo-dT column can be utilized in the fractionation. cDNA is synthesized from the poly-A tailed RNA thus obtained by a conventional method. The synthesis can be carried out by using a commercially available cDNA synthesis kit. DNA are amplified by PCR using the above-obtained cDNA as a template and primers designed and chemically synthesized on the basis of the nucleotide sequence of SEQ ID NO: 6. For example, when PCR is carried out by using cDNA derived from mustard (*Brassica juncea*) as the template and primers 33 (SEQ ID NO: 17) and 34 (SEQ ID NO: 18) shown in List 3 hereinafter, the genes from Cruciferae plants of the present invention, e.g., the "raffinose synthase gene having a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 5," and the "raffinose synthase gene having the nucleotide sequence represented by the 1st to 2654th nucleotides in the nucleotide sequence represented by SEQ ID NO: 6" can be obtained. According to a particular purpose, the PCR primers can also be designed and synthesized on the

basis of the nucleotide sequence of SEQ ID NO: 6. For example, in order to amplify DNA encoding the open reading frame region of the "raffinose synthase gene having a nucleotide sequence encoding a protein having the amino acid sequence of SEQ ID NO: 5", and the "raffinose synthase gene having the nucleotide sequence represented by the 134th to the 2467th nucleotides of SEQ ID NO: 6", preferably, oligonucleotides having the nucleotide sequences represented by primers 35 (SEQ ID NO: 19) and 36 (SEQ ID NO: 20) in List 3 are synthesized and used as the primers.

The amplified DNA can be cloned according to a conventional method, for example, described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; or "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X. Alternatively, cloning can be carried out, for example, by using a commercially available TA cloning kit (Invitrogen) or a commercially available plasmid vector such as pBluescript II (Stratagene). The nucleotide sequence of the DNA clone can be determined by dideoxy terminating method such as that described by F. Sanger, S. Nicklen, A.R. Coulson, Proceedings of National Academy of Science U.S.A. (1977), 74, pp. 5463-5467. For example, preferably, the commercially available ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer can be used.

List 3

Primer 31 (SEQ ID NO: 15): ttggaagaga agacgccgcc

gggaatcgtc 30mer

Primer 32 (SEQ ID NO: 16): ttaagccccg gcgagagctc tggccggaca 30mer

Primer 33 (SEQ ID NO: 17): accaatccaa aatctcatca aataatcgca 30mer

Primer 34 (SEQ ID NO: 18): aaataatagg ggcagtacaa attacaccac 30mer

Primer 35 (SEQ ID NO: 19): atggctccac cgagcgtaat taaatccga 29mer

Primer 36 (SEQ ID NO: 20): ctaaaactca tacttaatag aagacaaacc 30mer

Then, a nucleic acid having a partial nucleotide sequence of the gene of the present invention (hereinafter referred to as "the gene fragment") which is obtained by the above-described method is labeled and then used as a probe in a hybridization method. The probe can be hybridized to, for example, DNA derived from soybean, a Chenopdiaceae plant or a Cruciferae plant to detect a nucleic acid having the probe specifically bound thereto, thereby detecting a nucleic acid having the raffinose synthase gene.

As the DNA derived from soybean, a Chenopdiaceae plant such as beet or a Cruciferae plant such as mustard or rapeseed, for example, a cDNA library or a genomic DNA library of these plants can be used. The gene library may also be a commercially available gene library as such or a library constructed according to a conventional library construction method, for example,

described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X.

As the hybridization method, for example, plaque hybridization or colony hybridization can be employed, and they are selected depending upon the kind of vector used in the construction of a library. More specifically, when the library to be used is constructed with a phage vector, a suitable host microorganism is mixed with the phage of the library under infectious conditions to obtain transformants. The transformant is further mixed with a soft agar medium, and the mixture is plated on an agar medium. Thereafter, the mixture is cultured at 37°C until a plaque of an appropriate size appears. When the library to be used is constructed with a plasmid vector, the plasmid is introduced into a suitable host microorganism to form transformants. The transformant obtained is diluted to a suitable concentration and the dilution is plated on an agar medium, after which it is cultured at 37°C until a colony of an appropriate size appears. In either case of the above libraries, a membrane filter is placed on the surface of the agar medium after the above cultivation, so that the phage or transformant is transferred to the membrane. This membrane is denatured with an alkali, followed by neutralization, and for example, when a nylon membrane is used, the membrane is irradiated with ultraviolet light, so that DNA of the phage

or transformant is fixed on the membrane. This membrane is then subjected to a hybridization method wherein the gene fragment which has a partial nucleotide sequence of the gene of the present invention and labeled by a conventional method (hereinafter referred to as "the labeled gene fragment") is used as a probe. For this method, reference may be made, for example, to D.M. Glover ed., "DNA cloning, a practical approach" IRL PRESS (1985), ISBN 0-947946-18-7. There are various reagents and temperature conditions to be used in the hybridization. For example, in general, prehybridization is carried out by immersion of the membrane in a prehybridization solution [6 x SSC (0.9 M NaCl, 0.09 M citric acid), 0.1 to 1 (w/v)% SDS, 100 μg/ml denatured salmon sperm DNA] and incubation at 65°C for 1 hour. Then, hybridization is carried out by addition and mixing of the labeled gene fragment thereto and incubating the membrane at 42 to 68°C for 4 to 16 hours.

In the present invention, the "stringent conditions" are those wherein incubation is carried out, for example, at 65 to 68°C in the above hybridization.

After hybridization, the membrane is taken out and is washed with 2 x SSC containing 0.1 to 1 (w/v)% SDS, further rinsed with 0.2 x SSC containing 0.1 to 1 (w/v)% SDS, and then dried. The membrane is analyzed, for example, by autoradiography or other techniques to detect the position of the probe on the membrane, thereby detecting the position on the membrane of a nucleic acid having a nucleotide sequence homologous to that

of the probe used. The clone corresponding to the position of the nucleic acid thus detected on the membrane is identified on the original agar medium and the positive clone is selected so that the clone having the nucleic acid can be isolated. The same procedures of detection are repeated to purify the clone having the nucleic acid.

Alternatively, a commercially available kit such as GENE TRAPPER cDNA Positive Section System kit (GibcoBRL) can be used. In this method, first, a single-stranded DNA library is hybridized with the biotynylated gene fragment (i.e., probe), followed by adding streptoavidin-bound magnet beads and mixing. From the mixture, the streptoavidin-bound magnetic beads are collected with a magnet, so that single-stranded DNA having a nucleotide sequence homologous to that of the probe used, which has been bound to these beads through the gene fragment, biotin and streptoavidin, is collected and detected. The single-stranded DNA collected can be converted into a double-strand form by reaction with a suitable DNA polymerase using a suitable oligonucleotide as a primer.

As described above, a nucleic acid containing raffinose synthase gene can obtained by detecting a nucleic acid hybridizable to the gene fragment in DNAs of a gene library derived from soybean, a Chenopdiaceae plant or a Cruciferae plant, purifying a clone having the nucleic acid and isolating phage or plasmid DNA from the clone. By preparing the restriction map or determining the nucleotide sequence of the nucleic acid

thus obtained according to a conventional method, the nucleic acid containing the gene of the present invention can be confirmed.

For example, the gene of the present invention from a Chenopdiaceae plant can be confirmed by the following point:

The amino acid encoded by the nucleotide sequence thus determined has 75% or more homology to the amino acid sequence represented by the 103rd to 208th amino acids in the amino acid sequence of SEQ ID NO: 3;

80% or more homology to the amino acid sequence represented by the 255th to 271st amino acids in the amino acid sequence of SEQ ID NO:3;

70% or more homology to the amino acid sequence represented by the 289th to 326th amino acids in the amino acid sequence of SEQ ID NO: 3; or

70% or more homology to the amino acid sequence represented by the 610th to 696th amino acids in the amino acid sequence of SEQ ID NO: 3.

The gene of the present invention from a Cruciferae plant can be confirmed, for example, by the following point:

The amino acid sequence encoded by the nucleotide sequence determined has 75% or more homology to the amino acid sequence represented by the 111th to 213th amino acids in the amino acid sequence of SEQ ID NO: 5;

80% or more homology to the amino acid sequence represented by the 260th to 275th amino acids in the amino acid sequence of SEQ ID NO: 5;

70% or more homology to the amino acid sequence represented by the 293rd to 325th amino acids in the amino acid sequence of SEQ ID NO: 5; or

70% or more homology to the amino acid sequence represented by the 609th to 695th amino acids in the amino acid sequence of SEQ ID NO: 5.

The "homology" used herein means the proportion of the number of amino acids in a region, which are identical to those in a different region to be compared, to the number of the entire amino acids in the former region, upon comparing regions having similarity in two amino acid sequences. In this respect, it is preferred that the region having similarity contains more amino acids. Such homology of amino acid sequences can be evaluated by using a commercially available gene analysis software such as GENETIX (Software Kaihatu K.K.).

Further, according to the same manner as described above, a nucleic acid containing raffinose synthase gene can be detected by hybridization to DNA from the desired organism using the gene fragment as a probe to detect a nucleic acid to which the probe specifically binds (hereinafter referred to as the detection method of the present invention). The gene fragment used herein can be chemically synthesized according to a conventional method on the basis of the nucleotide sequence represented by SEQ ID NO: 2, 4, 6 or 8. Alternatively, it can be prepared by PCR using as primers oligonucleotides chemically synthesized according to a conventional method on the basis

of the nucleotide sequence represented by SEQ ID NO: 2, 4, 6 or 8.

The gene fragment may be a part of the non-translated region of the raffinose synthase gene as well as the open reading frame thereof. For example, an oligonucleotide having the same nucleotide sequence as a part of that of 5'-upstream side such as the 1st to 235th nucleotides in the nucleotide sequence of SEQ ID NO: 4, the 1st to 133rd nucleotides in the nucleotide sequence of SEQ ID NO: 6 and the like, or a part of that of 3'-downstream side such as the 2588th to 2675th nucleotides in the nucleotide sequence of SEQ ID NO: 4, the 2468th to 2676th nucleotides in the nucleotide sequence of SEQ ID NO: 6 and the like.

When PCR is carried out by using the gene fragment as primers, it is possible to amplify a nucleic acid containing raffinose synthase gene from DNA derived from the desired organism (hereinafter referred to as the amplification method of the present invention).

More specifically, for example, oligonucleotides having the nucleotide sequences of the gene fragment are designed and chemically synthesized according to a conventional method. In general, it is preferred that the number of nucleotides is more from a viewpoint that the specificity of annealing is ensured. It is, however, also preferred that the number of nucleotides is not so many from viewpoints that the primers themselves are liable to have a higher structure giving possible

deterioration of the annealing efficiency and that complicated procedures are required in the purification after the synthesis. Normally, oligonucleotides composed of 15 to 50 bases are preferred. In this respect, based on the codon table showing the correspondence of amino acids encoded by codons, a mixture of primers can also be synthesized by using a mixture of plural bases so that a residue at a specified position in a primer is changed to different bases according to the variation of codons which can encode one certain amino acid. Alternatively, for example, a base such as inosine which can form a base pair with plural bases can be used instead of the above mixture of plural bases.

Coding Table

Phe: UUU, UUC Ser: UCU, UCC, UCA, UCG, AGU, AGC

Tyr: UAU, UAC Cys: UGU, UGC

Stop: UAA, UAG, UGA Trp: UGG

Leu: UUA, UUG, CUU, CUC, CUA, CUG Pro: CCU, CCC, CCA, CCG His: CAU, CAC Gln: CAA, CAG Arg: CGU, CGC, CGA, CGG, AGA, AGG

Ile: AUU, AUC, AUA Thr: ACU, ACC, ACA, ACG

Asn: AAU, AAC Lys: AAA, AAG

Met: AUG

Val: GUU, GUC, GUA, GUG Ala: GCU, GCC, GCA, GCG

Asp: GAU, GAC Gly: GGU, GGC, GGA, GGG

Glu: GAA, GAG

In the above codon table, each codon is shown as the

nucleotide sequence in mRNA and its light hand is the 5'-terminus. U represents uracil base in RNA and corresponds to thymine base in DNA.

An oligonucleotide having the same nucleotide sequence as the coding strand of the double-stranded DNA of the gene of the present invention is called a "sense primer" and that having a nucleotide sequence complementary to the coding strand is called an "antisense primer".

A sense primer having the same nucleotide sequence as that of 5'-upstream side in the coding strand of the gene of the present invention, and an antisense primer having a nucleotide sequence complementary to the nucleotide sequence on the 3'-downstream side in this coding strand are used in combination for PCR reaction, for example, with a gene library, genomic DNA or cDNA as the template to amplify DNA. As the gene library to be used, for example, there are a cDNA library and a genomic library derived from soybean, a Chenopdiaceae plant such as beet or a Cruciferae plant such as mustard or rapeseed, etc. The gene library may also be a library constructed according to a conventional library construction method, for example, described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBN 0-471-50338-X, or a commercially available gene library as such. As the genomic DNA or cDNA, for example, there are those prepared from soybean, a Chenopdiaceae plant

such as beet or a Cruciferae plant such as mustard or rapeseed, etc. For example, PCR is carried out by using the primers 31 (SEQ ID NO: 15) and 32 (SEQ ID NO: 16) in the above List 3 and as the template cDNA derived from mustard to amplify DNA having the nucleotide sequence represented by the 749th to 1215th nucleotides in the nucleotide sequence of SEQ ID NO: 6. Further, PCR is carried out by using the primers and as the template cDNA derived from rapeseed to amplify DNA having the nucleotide sequence represented by the 1st to 467th nucleotides in the nucleotide sequence of SEQ ID NO: 8. The nucleic acid thus amplified can be confirmed by conventional electrophoresis. The nucleic acid can be cloned according a conventional method such as that described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor laboratory Press or "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc., ISBN 0-471-50338-X. For the nucleic acid, its restriction map is prepared or its nucleotide sequence is determined by a conventional method, so that the nucleic acid containing raffinose synthase gene or a part thereof can be identified. When the nucleic acid contains a part of raffinose synthase, PCR can be carried out on the basis of its nucleotide sequence to amplify the nucleic acid containing the 5'-upstream side nucleotide sequence or the 3'-downstream side nucleotide sequence. That is, based on the nucleotide sequence of the above-obtained nucleic acid, an antisense primer is designed and synthesized for amplification of the 5'-upstream side part,

and a sense primer is designed and synthesized for amplification of the 3'-downstream side part. The nucleotide sequence of the 5'-upstream side part or 3'-downsream side part of the nucleotide sequence already obtained can be determined by the RACE method using these primers and a commercially available kit such as Marathon Kit of Clontech. The full length raffinose synthase gene can be obtained by synthesizing new primers based on both terminal sequences in the nucleotide sequence thus determined and carrying out PCR again.

The above detection method of the present invention can also be used in the analysis of genotypes of a plant such as soybean, a Chenopdiaceae plant or a Cruciferae plant, etc. More specifically, for example, a genomic DNA derived from soybean, a Chenopdiaceae plant or a Cruciferae plant is prepared according to a conventional method, for example, described in "Cloning and Sequence (Plant Biotechnology Experiment Manual)" complied under the supervision of Itaru Watanabe, edited by Masahiro Sugiura, published by Noson Bunka-sha, Tokyo (1989). The genomic DNA is digested with at least several kinds of restriction enzymes, followed by electrophoresis. The electrophoresed DNA is blotted on a filter according to a conventional method. This filter is subjected to hybridization with a probe prepared from DNA having the gene fragment by a conventional method, and DNA to which the probe hybridizes is detected. The DNAs detected are compared in length between different varieties of a specified plant species. The

differences in length make possible the analysis of differences in phenotypic characteristics accompanied with the expression of raffinose family oligosaccharides between these varieties. Furthermore, when the DNAs detected by the above method are compared in length between the gene recombinant plant and the non-gene recombinant plant of the same variety, the former plant can be distinguished from the latter plant by the detection of hybridizing bands greater in number or higher in concentration for the former plant than for the latter plant. This method can be carried out according to the RFLP (restriction fragment length polymorphism) method, for example, described in "Plant PCR Experiment Protocols" complied under the supervision of Ko Shimamoto and Takuji Sasaki, published by Shujun-sha, Tokyo (1995), ISBN 4-87962-144-7, pp. 90-94.

Further, the amplification method of the present invention can be used for an analysis of genes of soybean, a Chenopdiaceae plant or a Cruciferae plant, etc. More specifically, for example, the amplification method of the present invention is carried out by using plant genomic DNA prepared from soybean, a Chenopdiaceae plant or a Cruciferae plant to amplify DNA. The amplified DNA is mixed with a formaldehyde solution, followed by heat denaturing at 85°C for 5 minutes and then quickly cooling on ice. A sample thereof is subjected to electrophoresis on, for example, 6 (w/v)% polyacrylamide gel containing 0 (v/v)% or 10 (v/v)% of glycerol. For this electrophoresis, a commercially available

electrophoresis apparatus such as that for SSCP (Single Strand Conformation Polymorhism) can be used and the electrophoresis can be carried out with maintaining the gel at a constant temperature, for example, at 5°C, 25°C, 37°C, etc. From the electrophoresed gel, DNA is detected, for example, by a method such as silver staining method with a commercially available reagent. From the differences of behavior between the varieties in the electrophoresis of the DNA detected, a mutation in the raffinose synthase gene is detected, and an analysis is carried out for differences caused by the mutation in phenotypic characteristics accompanied with the expression of raffinose family oligosaccharides. This method can be carried out according to the SSCP method, for example, described in "Plant PCR Experiment Protocols" complied under the supervision of Ko Shimamoto and Takuji Sasaki, published by Shujun-sha, Tokyo (1995), ISBN 4-87962-144-7, pp. 141-146.

The analysis of the plant gene from soybean, a Chenopdiaceae plant or a Cruciferae plant by the above detection method or amplification method of the present invention can be used not only for the analysis of differences in phenotypic characteristics accompanied with the expression of raffinose family oligosaccharides, but also, for example, for the selection of clones having the desired characters upon production of a novel variety of soybean, a Chenopdiaceae plant or a Cruciferae plant. Further, it can also be used for identification of a clone thus produced and having the characters derived from a

recombinant plant upon producing a plant variety using the recombinant plant.

For expression of the gene of the present invention in cells of a host, preferably, a nucleic acid comprising a nucleic acid fragment which contains the gene of the present invention, and a nucleic acid fragment which has a promoter activity in the host cells and joined to the former nucleic acid fragment (hereinafter referred to as the expression nucleic acid of the present invention) can be used.

The nucleic acid fragment having promoter activity in the expression nucleic acid of the present invention is not limited to a specific one, so long as it is functionable in a host to be transformed. For example, there are synthetic promoters functionable in *Escherichia coli*, such as *E. coli* lactose operon promoter, *E. coli* tryptophan operon promoter and tac promoter, etc.; yeast alcohol dehydrogenase gene (ADH) promoter, adenovirus major late (Ad.ML) promoter, SV40 early promoter, baculovirus promoter and the like. When the host is a plant, the promoter may include, for example, T-DNA derived constitutive promoters such as nopaline synthase gene (NOS) promoter, octopine synthase gene (OCS) promoter, etc.; plant virus-derived promoters such as cauliflower mosaic virus (CaMV)-derived 19S and 35S promoters; inducible promoters such as phenylalanine ammonia-lyase (PAL) gene promoter, chalcone synthase (CHS) gene promoter, pathogenesis-related protein (PR) gene promoter, etc. Furthermore, vector pSUM-GY1 (see JP-A

06-189777/1994) can also be used, which has a promoter giving specific expression in a specified plant tissue, e.g., a promoter of soybean-derived seed storage protein glycinin gene (JP-A 6-189777).

Furthermore, a nucleic acid fragment having a terminator activity can be joined to the expression nucleic acid of the present invention. In this case, it is generally preferred that the expression nucleic acid of the present invention is constructed so that the nucleic acid fragment having a terminator activity is positioned downstream the raffinose synthase gene. The terminator to be used is not particularly limited, so long as it is functionable in cells of a host to be transformed. For example, when the host is a plant, there are T-DNA derived constitutive terminators such as nopaline synthase gene (NOS) terminator, etc.; plant derived terminators such as terminators of allium virus GV1 or GV2, and the like.

The expression nucleic acid of the present invention can be introduced into a host cell according to a conventional gene engineering technique to obtain a transformant. If necessary, the expression nucleic acid of the present invention can be inserted into a vector having a suitable marker depending upon a particular transformation technique for introduction of the nucleic acid into a host cell.

A vector into which the expression nucleic acid of the present invention is inserted can be introduced into a microorganism according to a conventional method, for example,

described in "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor laboratory Press or "Current Protocols in Molecular Biology" (1987), John Wiley & Sons, Inc., ISBN 0-471-50338-X. The microorganism transformed with the vector can be selected on the basis of a selection marker such as antibiotic resistance, auxotrophy or the like. In case that the gene of the present invention is joined to the downstream of an inducible promoter (e.g., tac promoter) in the translatable form in the selected microorganism (e.g., *E. coli* transformant), a translated product of the gene of the present invention can be expressed under conventional culture and inducible conditions and can be recover as a peptide or a protein.

The raffinose synthase activity of the translated product of the gene of the present invention thus prepared can be measured by, for example, a method described in L. Lehle and W. Tanner, Eur. J. Biochem., 38, 103-110 (1973) to identify the translated product having the "capability of binding D-galactosyl group through α (1→6) bond to the hydroxyl group attached to the carbon atom at 6-position of the D-glucose residue in sucrose molecule". More specifically, for example, the gene of the present invention is cloned in pGEX-4T3 (Pharmacia) to obtain a plasmid containing the expression nucleic acid of the present invention. The resultant plasmid is introduced into, for example, *E. coli* HB101 strain to obtain a transformant. The resultant transformant is culture overnight and 1 ml of the culture is inoculated into 100 ml of LB culture medium.

It is incubated at 37°C for about 3 hours and IPTG (isopropylthio-β-D-galactoside) is added at a final concentration of 1 mM, followed by further incubation for 5 hours. Cells are recovered from the culture broth by centrifugation and are suspended by addition of 10 times of the cell weight of 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM DTT (dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM benzamide. The suspension is sonicated with a ultrasonic disrupter (Branson) to disrupt the cells. The disrupted cell suspension is centrifuged to recover a soluble protein solution. The resultant protein solution is added to a reaction mixture containing at final concentrations of 100 mM Tris-HCl (pH 7.4), 5 mM DTT (dithiothreitol), 0.01% BSA, 200 μM sucrose, 5 mM galactinol and 31.7 μM [^{14}C] sucrose. The reaction mixture is incubated at 37°C, followed by addition of 1.5 times in volume of ethanol and stirring. Insoluble materials are remove by centrifugation, the supernatant is spotted on, for example, a HPTLC cellulose thin layer chromatography plate (Merch, HPTLC plates cellulose) and then the plate is developed with n-butanol-pyridine-water-acetic acid (60 : 40 : 30 : 3). The developed plate is dried and analyzed with an imaging analyzer (FUJIX Bio-Image Analyzer BAS-2000II manufactured by Fuji Film) to determine [^{14}C] raffinose produced to measure the raffinose synthase activity.

In addition, the translated product as prepared above can also be used as an antigen for producing an antibody. The

antibody thus produced can be used for, for example, detection and determination of the gene of the present invention in a crude protein extract prepared from an organism such as a plant.

When the host is a plant, the vector into which the gene of the present invention is inserted can be introduced into plant cells by a conventional means such as *Agrobacterium* infection method (JP-B 2-58917 and JP-A 60-70080), electroporation into protoplasts (JP-A 60-251887 and JP-B 5-68575) or particle gun method (JP-A 5-508316 and JP-A 63-258525). The plant cell transformed by the introduction of the vector can be selected on the basis of a selection marker, for example, resistance to an antibiotic such as kanamycin or hygromycin. From the plant cell thus transformed, a transformed plant can be regenerated by a conventional plant cell cultivation method, for example, described in "Plant Gene Manipulation Manual (How to Produce Transgenic Plants)" written by Uchimiya, 1990, Kodan-sha Scientific (ISBN 4-06-153513-7), pp. 27-55. Furthermore, the collection of seeds from the transformed plant also makes it possible to prolify the transformed plant. In addition, crossing between the transformed plant obtained and the non-transformed plant makes it possible to produce progenic plants with the characters of the transformed plant.

As gene engineering techniques in soybean, basically, the above general techniques can be employed. More specifically, "transformation of soybean plant strain by particle gun" described in EP 301749, gene introduction methods. for example,

described in Torisky, R.S., Kovacs, L., Avdiushko, S., Newman, J.D., Hunt, A.G. and Collins, G.B., "Development of a binary vector system for plant transformation based on the supervirulent Agrobacterium tumefaciens strain Chry5", Plant Cell Rep., (1997), 17, p. 102-108, etc. can be employed.

As gene engineering techniques in a Chenopdiaceae plant, basically, the above general techniques can be employed. More specifically, gene introduction methods, for example, described in M. Mannerlof, S. Tuvesson, P. Steen and P. Tenning, "Transgenic sugar beet tolerant to glyphosate", Euphytica (1997), 94, p 83-91, B.K. Konwar, "Agrobacterium tumefacience-Mediated Genetic Transformation of Sugar Beet (Beta vulgaris L.)", J. Plant Biochemistry & Biotechnology (1994), 3, p. 37-41 can be employed.

As gene engineering techniques in a Cruciferae plant, basically, the above general techniques can be employed. More specifically, the gene introduction can be carried out according to a method, for example, described in J. Fry, A. Barnason and R.B. Horsch, "Transformation of Brassica napus with Agrobacterium tumefaciens based vectors", Plant Cell Reports (1987), 6, 321-325.

For example, when gene introduction is carried out by *Agrobacterium* infection method, first, the above-described expression nucleic acid of the present invention is inserted into a binary vector. The resultant vector can be introduced into, for example, *Agrobacterium tumefaciens* LBA 4404 strain

which has been converted into a competent state by treatment with calcium chloride. A transformant can be selected by an appropriate selection method according to the selection marker gene of the vector, for example, cultivation of a strain containing the vector in a culture medium containing an antibiotic in case that the selection marker gene is that giving resistance to the antibiotic such as kanamycin. The resultant transformed *Agrobacterium* strain can be culture in a liquid culture medium, for example, LB medium.

Soybean, a Chenopdiaceae plant or a Cruciferae plant can be transformed by using thus obtained *Agrobacterium* transformant culture broth as described below. For example, seeds from soybean, beet, rapeseed or mustard is sowed aseptically in, for example, 1/2 MS medium containing 2% sucrose and 0.7% agar. After about 1 week, cotyledons and petioles of the germinated plant are cut off with a scalpel aseptically and transplanted in, for example, MS medium containing 3% sucrose, 0.7% agar, 4.5 μM BA, 0.05 μM 2,4-D and 3.3 μM $AgNO_3$ and cultured for one day. The cotyledons and petioles thus precultured are transferred to 1000-fold dilution of the above *Agrobacterium* culture broth and allowed to stand for 5 minutes. The cotyledons and petioles are transferred to the same medium as that of the preculture again and cultured for about 3 to 4 days. The cotyledones and petioles thus cultured are transferred to, for example, MS medium containing 3% sucrose, 4.5 μM BA, 0.05 μM 2,4-D, 3.3 μM $AgNO_3$ and 500 mg/liter cefotaxim, followed by

shaking for 1 day to remove microbial cells. The resultant cotyledons and petioles are transferred to, for example, MS medium containing 3% sucrose, 0.7% agar, 4.5 μM BA, 0.05 μM 2,4-D, 3.3 μM $AgNO_3$ 100 mg/liter cefotaxim and 20 mg/liter kanamycin, followed by culturing for 3 to 4 weeks. Then, the cotyledons and petioles are transferred to, for example, MS medium containing 3% sucrose, 0.7% agar, 4.5 μM BA, 0.05 μM 2,4-D, 100 mg/liter cefotaxim and 20 mg/liter kanamycin and cultured. Culture in this medium is continued with subculturing every 3 to 4 weeks. When a shoot are regenerated, it is subcultured in, for example, MS medium containing 3% sucrose, 0.7% agar and 20 mg/liter kanamycin for 3 to 4 weeks. When the plant makes roots, it is transferred to vermiculite-peat moss (1 : 1) and acclimatized by culturing at 21 to 22°C under day and night conditions of 12 hours: 12 hours = day time : night. As the plant grows, it is transferred to appropriate cultivation soil to culture the plant. A genomic DNA is extracted from the leaf of the regenerated plant according to the above method and PCR is carried out by using as primers having partial nucleotide sequences of the expression nucleic acid of the present invention to confirm the insertion of the gene of the present invention into the plant.

As described hereinabove, by introducing the gene of the present invention into a plant, for example, soybean, a Chenopdiaceae plant or a Cruciferae plant, it is possible to vary the expression level and activity of raffinose synthase

in the plant to control the amount of raffinose family oligosaccharides in the plant. The gene of the present invention is useful in techniques for varying the expression level and activity of raffinose synthase in soybean, a Chenopdiaceae plant or a Cruciferae plant on the basis of gene homology, for example, techniques such as homologous recombination and antisense technique, cosuppression and the like.

The following examples further illustrate the present invention in detail but are not to be construed to limit the scope of the present invention.

Example 1

Preparation of cDNA Derived from Soybean

About 2 g of immature seeds of soybean (*Glycine max*) Williams82 were frozen in liquid nitrogen and then ground with a mortar, to which 20 ml of Isogen (Nippon Gene) was added, and the mixture was further thoroughly ground. The ground material was transferred into a centrifugation tube, to which 4 ml of chloroform was added, and the mixture was stirred with a vortex mixer and then centrifuged at 6,500 x g for 10 minutes at 4°C. The water layer was collected, to which 10 ml of isopropanol was added, and the mixture was stirred and then centrifuged at 6,500 x g for 10 minutes at 4°C. The resulting precipitate was washed with 10 ml of 70% ethanol and then dissolved in 1 ml of elution buffer (10 mM Tris-HCl/pH 7.5, 1 mM EDTA, 0.1% SDS). The solution was allowed to stand at 60°C for 10 minutes and then centrifuged at 10,000 x g for 1 minute to remove insoluble

matter. To the resulting supernatant was added an equivalent volume of Oligotex-dT30 (Takara), and the mixture was stirred and then allowed to stand at 65°C for 5 minutes. Further, the mixture was placed on ice and allowed to stand for 3 minutes, to which 200 μl of 5 M NaCl was added, and the mixture was mixed and then allowed to stand at 37°C for 10 minutes. The mixture was then centrifuged at 10,000 x g for 3 minutes at 4°C. The precipitate was collected and then suspended in 1 ml of TE buffer, and the suspension was allowed to stand at 65°C for 5 minutes. Further, the suspension was placed on ice and then allowing to stand for 3 minutes, followed by centrifugation at 10,000 x g for 3 minutes at 4°C to remove precipitate.

To the resulting supernatant were added 100 μl of 3M sodium acetate and 2 ml of ethanol to precipitate and collect RNA. The collected RNA was washed twice with 70% ethanol and then dissolved in 20 μl of sterilized water, which was used for the subsequent cDNA synthesis. The amount of RNA obtained was determined by the measurement of absorbance at 260 nm.

For the cDNA synthesis, First Strand Synthesis Kit for RT-PCR (Amersham) and cDNA Synthesis Kit (Takara) were used, and all operations were made according to the protocol attached to kits.

Example 2

Cloning of Raffinose Synthase Gene from Soybean cDNA

PCR was carried out by using the cDNA obtained from

immature seeds of soybean (*Glycine max*) Williams82 in Example 1 as a template and the primers designed on the basis of the amino acid sequence of SEQ ID No: 1, i.e., primers having nucleotide sequences shown in List 4 (SEQ ID NOS: 21 and 22) below to amplify a DNA fragment. The PCR was carried out with Gene Amp PCR Systems 2400 and DNA Thermal Cycler Model 480 of Perkin-Elmer using Advantage KlenTaq cDNA Kit of Clontech. The reaction was carried out by repeating the cycle for maintaining at 94°C for 1 minute, at 50°C for 3 minutes and then at 72°C for 3 minutes 40 times to amplify the DNA fragment. The amplified DNA fragment was cloned with TA cloning kit (Invitrogen), followed by sequence reaction using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer and analysis of the nucleotide sequence with a 373S DNA sequencer of ABI. Based on this sequence, primers having nucleotide sequences shown in List 5 below were synthesized. The synthesis of cDNA was carried out with Marathon Kit of Clontech using mRNA obtained from leaves of soybean Williams82 in Example 1. The cDNA obtained was ligated to an adapter contained in the kit with ligase. These operations were carried out according to the protocol attached to the kit. By using the adapter-ligated cDNA thus prepared, PCR was carried out with the primers shown in List 5 (SEQ ID NO: 23) according to the same manner as the above. The nucleotide sequence in terminal region of the gene was analyzed according to the protocol attached to the Marathon Kit of Clontech. As a result, the nucleotide sequence of SEQ ID NO: 2 was determined.

List 4

4-5-F primer (SEQ ID NO: 21):

cgatggatgg giaaittiat icaiccigai tgggaiatgt t 41mer

4-6-RV primer (SEQ ID NO: 22):

ggccacatit tiacia(ag)icc iatiggigci aa 32mer

List 5

5-SC-2 (SEQ ID NO: 23):

tgttactagg cgaaacaaga gtagctctga 30mer

Example 3

Preparation of cDNA derived from Chenopdiaceae Plant

About 2 g of leaves of beet (*Beta vulgaris*: haming) was frozen in liquid nitrogen and then ground with a mortar, to which 20 ml of Isogen (Nippon Gene) was added, and the mixture was further thoroughly ground. The ground material was transferred into a centrifugation tube, to which 4 ml of chloroform was added, and the mixture was stirred with a vortex mixer and then centrifuged at 6,500 x g for 10 minutes at 4°C. The water layer was collected, to which 10 ml of isopropanol was added, and the mixture was stirred and then centrifuged at 6,500 x g for 10 minutes at 4°C. The resulting precipitate was washed with 10 ml of 70% ethanol and then dissolved in 180 μl of DEPC-treated sterilized water. The solution was allowed to stand at 55°C for 5 minutes and 20 μl of 5 M NaCl was added thereto. The resulting solution was purified using BIOMAG mRNA PURIFICATION KIT (PerSeptive Biosystems: Catalog No. 8-MB4003K).

To the resulting mRNA solution were added 3M sodium

acetate and ethanol, and RNA was precipitated and collected. The collected RNA was washed twice with 70% ethanol and then dissolved in 20 μl of sterilized water, which was used for the subsequent cDNA synthesis. The amount of RNA obtained was determined by the measurement of absorbance at 260 nm.

For the cDNA synthesis, SMART PCR cDNA Synthesis Kit (Clontech) was used, and all operations were made according to the protocol attached to the kit.

Example 4

Analysis of Nucleotide Sequence of Raffinose Synthase Gene from Chenopdiaceae Plant

Synthetic DNA primers having the nucleotide sequences shown in List 6 (SEQ ID NOS: 24-27) below were synthesized. The PCR method was carried out with Gene Amp PCR Systems 2400 and DNA Thermal Cycler Model 480 of Perkin-Elmer using Advantage KlenTaq cDNA Kit of Clontech. The PCR was carried out with the above primers (SEQ ID NOS: 24-27) and cDNA of beet obtained in the above Example 3 by repeating the cycle for maintaining at 94°C for 1 minute, at 50°C for 3 minutes and then at 72°C for 3 minutes 40 times. As a result, the combinations of primers 6-3-F (SEQ ID NO: 24) and 6-8-RV (SEQ ID NO: 25) and primers 6-10-F (SEQ ID NO: 26) and 6-6-RV (SEQ ID NO: 27) gave an amplification of about 0.3 kb and 0.6 kb bands, respectively. The amplified DNA fragments were cloned with TA cloning kit (Invitrogen), followed by sequence reaction using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer

and analysis of nucleotide sequence with a 373S DNA sequencer of ABI. Based on the resulting nucleotide sequences, synthetic DNA primers having nucleotide sequences shown in List 7 (SEQ ID NOS: 28-35) below were prepared and PCR was carried out using cDNA obtained from beet in Example 3 in the same manner as above. As a result, DNA having the nucleotide sequence of SEQ ID NO: 4 was finally obtained from cDNA of beet.

List 6

6-3-F (SEQ ID NO: 24):

cgaggiggit gicciccigg ittigtiati atigaigaig gitggca 47mer

6-8-RV (SEQ ID NO: 25):

at(t/c)tt(a/g)tcia cigcia(a/g)(a/g)tc (t/c)tccatigt 29mer

6-10-F (SEQ ID NO: 26):

ggiacitait gg(c/t)ticaigg itgicaiatg gticaitg 38mer

6-6-RV (SEQ ID NO: 27):

ggccacatit tiacia(a/g)icc iatiggigci aa 32mer

List 7

7-Sb-1 (SEQ ID NO: 28):

atctatttgt catgacgatg atccga 26mer

7-Sb-2RV (SEQ ID NO: 29):

ggccctcatt cccatattgg gatgatcctc 30mer

7-Sb-3RV (SEQ ID NO: 30):

aagcatgcca aacatacaca tgctcaacag 30mer

7-Sb-4RV (SEQ ID NO: 31):

agacccgggg aaagctttgg ggttactact 30mer

7-Sb-5 (SEQ ID NO: 32):

tggatgggaa actttataca ccctgact 28mer

7-Sb-6 (SEQ ID NO: 33):

gacatgttcc catctacaca cccttgtg 28mer

7-Sb-7 (SEQ ID NO: 34):

ccaatttatg ttagtgatgt tgttggcaag 30mer

7-Sb-8RV (SEQ ID NO: 35):

tcgactccca gggtagaatt gtcatc 26mer

Example 5

Preparation of cDNA Derived from Cruciferae Plant

About 2 g of leaves of mustard (*Brassica juncea*) was frozen in liquid nitrogen and then ground with a mortar, to which 20 ml of Isogen (Nippon Gene) was added, and the mixture was further thoroughly ground. The ground material was transferred into a centrifugation tube, to which 4 ml of chloroform was added, and the mixture was stirred with a vortex mixer and then centrifuged at 6,500 x g for 10 minutes at 4°C. The water layer was collected, to which 10 ml of isopropanol was added, and the mixture was stirred and then centrifuged at 6,500 x g for 10 minutes at 4°C. The resulting precipitate was washed with 10 ml of 70% ethanol and then dissolved in 180 μl of DEPC-treated sterilized water. The solution was allowed to stand at 55°C for 5 minutes and to which 20 μl of 5 M NaCl was added. The resulting solution was purified using BIOMAG mRNA PURIFICATION KIT (PerSeptive Biosystems: Catalog No. 8-MB4003K).

To the resulting mRNA solution were added 3M sodium acetate and ethanol, and RNA was precipitated and collected.

The collected RNA was washed twice with 70% ethanol and then dissolved in 20 μl of sterilized water, which was used for the subsequent cDNA synthesis. The amount of RNA obtained was determined by the measurement of absorbance at 260 nm.

For the cDNA synthesis, SMART PCR cDNA Synthesis Kit (Clontech) was used, and all operations were carried out according to the protocol attached to the kit.

In the same manner as described in the above, mRNA was purified from immature seeds of rapeseed Westar (*Brassica napus*) and cDNA was synthesized.

Example 6

Isolation and Nucleotide Sequence Analysis of Raffinose Synthase Gene derived from Cruciferae Plant

DNA primers having the nucleotide sequences shown in List 8 (SEQ ID NOS: 36 and 37) below were synthesized. PCR was carried out with Gene Amp PCR Systems 2400 and DNA Thermal Cycler Model 480 of Perkin-Elmer using Advantage KlenTaq cDNA Kit of Clontech. The PCR was carried out with the above primers and cDNA of mustard obtained in Example 5 by repeating the cycle for maintaining at 94°C for 1 minute, at 50°C for 3 minutes and then at 72°C for 3 minutes 40 times. The reaction products were analyzed by agarose gel electrophoresis. As a result, an amplification of about the 1.2 kb bands was detected. The amplified DNA fragment was cloned with TA cloning kit (Invitrogen), followed by sequence reaction using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit of Perkin-Elmer and nucleotide

sequence analysis with a 373S DNA sequencer of ABI. Based on the resulting nucleotide sequence, synthetic primers having the nucleotide sequences shown in List 9 (SEQ ID NOS: 38 and 39) below were prepared and PCR was carried out using cDNAs from mustard (*Brassica juncea*) and rapeseed Westar (*Brassica napus*) obtained in Example 5 according to the same manner as the above. As a result, the nucleotide sequence represented by the 749th to 1215th nucleotides of SEQ ID NO: 6 and by the 1st to 467th nucleotides of SEQ ID NO: 8 were finally determined for cDNA from mustard (*Brassica juncea*) and rapeseed Westar (*Brassica napus*), respectively.

List 8

8-#1 (SEQ ID NO: 36):

cgattiaaig titggtggac iacicaitgg gtigg 35mer

8-#10RV (SEQ ID NO: 37):

caitgiacca titgicaicc itgia(ag)ccai taigticc 38mer

List 9

9-primer-1 (SEQ ID NO: 38):

gttagggttc atatgaacac cttcaagctc 30mer

9-primer-2RV (SEQ ID NO: 39):

caacggcgag atcttgcatc gtcaac 26mer

Example 7

Nucleotide Sequence Analysis of Raffinose Synthase Full-Length Gene Derived from Cruciferae Plant

Based on the nucleotide sequences obtained in Example 6, DNA primers having the nucleotide sequences shown in List

10 (SEQ ID NOS: 40-46) below were synthesized. The cDNAs from mustard (*Brassica juncea*) and rapeseed Westar (*Brassica napus*) obtained in the same manner as described in Example 5 were ligated to adapters contained in Marathon Kit of Clontech. By using the adapter-ligated cDNAs thus prepared, PCR was carried out with primers shown in List 10. 10-B-2RV (SEQ ID NO: 40), 10-B-3RV (SEQ ID NO: 41) and 10-B-4RV (SEQ ID NO: 42) primers were used for nucleotide analysis of 5'-termini, and 10-B-1 (SEQ ID NO: 43), 10-B-8 (SEQ ID NO: 44), 10-B-7 (SEQ ID NO: 45) and 10-B-6 (SEQ ID NO: 46) primers were used for nucleotide analysis of 3'-termini. The nucleotide sequences were analyzed according to the protocol attached to the Marathon Kit of clontech. As a result, the nucleotide sequence of SEQ ID NO: 6 and SEQ ID NO: 8 were determined from mustard (*Brassica juncea*) and rapeseed Westar (*Brassica napus*), respectively.

List 10

10-B-2RV (SEQ ID NO: 40):

ggattcgaca caaaccgcca cgtcatcgtc 30mer

10-B-3RV (SEQ ID NO: 41):

ccacgtgcac cacccgaact tatcgac 27mer

10-B-4RV (SEQ ID NO: 42):

aacatcgata ccatcggagt catgtccaat 30mer

10-B-1 (SEQ ID NO: 43):

gttagggttc atatgaacac cttcaagctc 30mer

10-B-8 (SEQ ID NO: 44):

tctacgtctg gcacgcgctt tgcggctac 29mer

10-B-7 (SEQ ID NO: 45):

gttgacgtca tccacatatt ggagatgttg t 31mer

10-B-6 (SEQ ID NO: 46):

gttatcgcta gcatggagca ctgtaatga 29mer

Example 8

Construction of Expression Vectors in Plant for Raffinose Synthase Gene Derived from Cruciferae Plant

Based on the nucleotide sequence of raffinose synthase gene from mustard obtained in Example 7, DNA primers having the nucleotide sequences shown in List 11 (SEQ ID NOS: 47 and 48) were prepared. By using cDNA of mustard, PCR was carried out in the same manner as described in Example 6. The amplified DNA fragment was digested with SacI. The DNA fragment thus digested was ligated to the vector pBI121(-) previously digested with SacI by using Ligation Kit (Takara). Plasmid pBI121 (Clontech) were digested with BamHI and SacI, and ligated to linkers shown in List 12 (SEQ ID NOS: 49 and 50) to prepare the vector pBI121(-). The vector thus obtained was analyzed by a restriction map and PCR using primers having nucleotide sequences shown in List 13 (SEQ ID NOS: 51-53), and confirmed the direction of inserted raffinose synthase gene. The vector whose raffinose synthase gene from mustard was inserted in the expressible direction was designated BjRS-Sac(+)-121 and the one whose raffinose synthase gene from mustard was inserted in the reverse direction was designated BjRS-Sac(-)-121.

List 11

11-SacI-BjN (SEQ ID NO: 47):

aacgagctca atccaaaatc tcatcaaata atcgc 35mer

11-SacI-BjintRV (SEQ ID NO: 48):

acaatagttg agggcggaag agtag 25mer

List 12

12-BamSac-(+)linker (SEQ ID NO: 49):

gatcgagctc gtgtcggatc cagct 25mer

12-BamSac-(-)linker (SEQ ID NO: 50):

ggatccgaca cgagctc 17mer

List 13

13-35S-3 (SEQ ID NO: 51):

cctcctcgga ttccattgcc cagctatctg 30mer

13-B-2RV (SEQ ID NO: 52):

ggattcgaca caaaccgcca cgtcatcgtc 30mer

13-B-8 (SEQ ID NO: 53):

tctacgtctg gcacgcgctt tgcggctac 29mer

Example 9

Transformation with Raffinose Synthase Gene Derived from Cruciferae Plant

The vectors BjRS-Sac(+)-121 and BjRS-Sac(-)-121 prepared in Example 8 were used for the transformation of mustard (*Brassica juncia*) by the *Agrobacterium* infection method.

Agrobacterium tumefacients (strain LBA4404 having rifampicin and streptomycin resistance) previously converted into a competent state by calcium chloride treatment was transformed independently with two plasmids BjRS-Sac(+)-121

and BjRS-Sac(-)-121 prepared in Example 8. The transformants were selected on LB medium containing 50 μg/ml rifampicin and 25 μg/ml kanamycin by utilizing the kanamycin resistant character conferred by the kanamycin resistant gene (neomycin phosphotransferase, NPTII) of the introduced plasmids.

The transformant Agrobacterium obtained (*Agrobacterium tumefaciens* strain LBA4404: rifampicin and streptomycin resistant) was cultured on LB medium containing 50 μg/ml rifampicin and 25 μg/ml kanamycin at 28°C for a whole day and night, and the culture was used for the transformation of mustard by the method described below.

The seeds of mustard were aseptically sowed on 1/2 MS medium containing 2% sucrose and 0.7% agar. After one week, cotyledons and petioles of sprouting plants were cut out with a scalpel, and transferred to MS medium containing 3% sucrose, 0.7% agar, 4.5 μM BA, 0.05 μM 2.4-D and 3.3 μM $AgNO_3$, followed by preculture for 1 day. The precultured cotyledons and petioles were transferred in a 1000-fold dilution of the Agrobacterium culture broth and allowed to stand for 5 minutes. The cotyledons and petioles were transferred again to the same medium as used in the preculture, and cultured for 3 to 4 days. The cultured cotyledons and petioles were transferred to MS medium containing 3% sucrose, 4.5 μM BA, 0.05 μM 2.4-D, 3.3 μM $AgNO_3$ and 500 mg/l cefotaxim, and shaken for 1 day to remove microbial cells. The cotyledons and petioles thus treated were transferred to MS medium containing 3% sucrose, 0.7% agar, 4.5 μM BA, 0.05

μM 2.4-D, 3.3 μM $AgNO_3$, 100 mg/l cefotaxim and 20 mg/l kanamycin, and cultured for 3 to 4 weeks. The cotyledons and petioles were transferred to MS medium containing 3% sucrose, 0.7% agar, 4.5 μM BA, 0.05 μM 2.4-D, 100 mg/l cefotaxim and 20 mg/l kanamycin, and cultivated. The cultivation on this medium was continued with subculturing at intervals of 3 to 4 weeks. When shoots are began to regenerate, these shoots are subcultured on MS medium containing 3% sucrose, 0.7% agar and 20 mg/l kanamycin, and cultivated for 3 to 4 weeks. The rooting plants are transferred to vermiculite : peat moss = 1 : 1, and cultivated at 21°C to 22°C in a cycle of day/night = 12 hours : 12 hours. With the progress of plant body growth, the plants are grown with cultivation soil.

BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO: 1 shows an amino acid sequence of a raffinose synthase protein encoded by the raffinose synthase gene of the present invention.

SEQ ID NO: 2 shows a nucleotide sequence of the raffinose synthase gene of the present invention.

SEQ ID NO: 3 shows an amino acid sequence of a raffinose synthase protein encoded by the raffinose synthase gene obtained from beet.

SEQ ID NO: 4 shows a cDNA nucleotide sequence of the raffinose synthase gene obtained from beet.

SEQ ID NO: 5 shows an amino acid sequence of a raffinose

synthase protein encoded by the raffinose synthase gene obtained from mustard.

SEQ ID NO: 6 shows a cDNA nucleotide sequence of the raffinose synthase gene obtained from mustard.

SEQ ID NO: 7 shows an amino acid sequence of a raffinose synthase protein encoded by the raffinose synthase gene obtained from rapeseed.

SEQ ID NO: 8 shows a cDNA nucleotide sequence of the raffinose synthase gene obtained from rapeseed.

List 1:

The nucleotide sequences shown in List 1 (SEQ ID NOS: 9 and 10) are examples of the typical primers used in the amplification of a DNA fragment having a raffinose synthase gene. All of these sequences are based on the nucleotide sequence of SEQ ID NO: 2. Primer 11 (SEQ ID NO: 9) is a sense primer and Primer 12 (SEQ ID NO: 10) is an antisense primer. Depending upon the purpose, recognition sequences for suitable restriction enzymes can be added to the 5'-termini of these nucleotide sequences.

List 2:

The nucleotide sequences shown in List 2 (SEQ ID NOS: 11-14) are examples of the typical primers used in the amplification of a cDNA of a raffinose synthase gene. Primer 21 (SEQ ID NO: 11) is a sense primer corresponding to the 5'-terminus of the beet-derived raffinose synthase gene. Primer 22 (SEQ ID NO: 12) is an antisense primer corresponding to the 3'-terminus.

Depending upon the purpose, recognition sequences for suitable restriction enzymes can be added to the 5'-termini of these nucleotide sequences.

Primer 23 (SEQ ID NO: 13) is a sense primer corresponding to the N-terminus of the open reading frame, and primer 24 (SEQ ID NO: 14) is an antisense primer corresponding to the C-terminus.

List 3:

Among the nucleotide sequences shown in List 3 (SEQ ID NOS: 15-20), primers 31 (SEQ ID NO: 15) and 32 (SEQ ID NO: 16) are typical primers used in the amplification of a DNA having the partial nucleotide sequence of a raffinose synthase gene. Primer 31 (SEQ ID NO: 15) is a sense primer used in the amplification of a DNA having the partial nucleotide sequence of a raffinose synthase gene from mustard and rapeseed and primer 32 (SEQ ID NO: 16) is an antisense primer. Depending upon the purpose, recognition sequences for suitable restriction enzymes can be added to the 5'-termini of these nucleotide sequences.

Primers 33 (SEQ ID NO: 17) and 34 (SEQ ID NO: 18) are the typical primers used in the amplification of a cDNA of a raffinose synthase gene of mustard. Primers 33 (SEQ ID NO: 17) and 34 (SEQ ID NO: 18) are both based on the nucleotide sequence of raffinose synthase gene in the non-translated region. Primer 33 (SEQ ID NO: 17) is a sense primer corresponding to the 5'-terminal non-translated region of the mustard-derived raffinose synthase gene. Primer 34 (SEQ ID NO: 18) is an antisense primer corresponding to the 3'-terminal non-translated region.

Primers 35 (SEQ ID NO: 19) and 36 (SEQ ID NO: 20) are typical primers used in the amplification of an open reading frame coding for the amino acid sequence of a raffinose synthase protein in the cDNA of a raffinose synthase gene. Primer 35 (SEQ ID NO: 19) is a sense primer corresponding to the 5'-terminus of the above open reading frame. Primer 36 (SEQ ID NO: 20) is an antisense primer corresponding to the 3'-terminus. Depending upon the purpose, recognition sequences for suitable restriction enzymes can be added to the 5'-termini of these nucleotide sequences.

List 4:

The nucleotide sequences shown in List 4 (SEQ ID NOS: 21 and 22) are of the primers used in the cloning of a DNA fragment having the present raffinose synthase gene. The base represented by the symbol "i" is inosine. The bases shown in parentheses mean that a mixture of those bases is used in the synthesis. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 5:

The nucleotide sequence shown in List 5 (SEQ ID NO: 23) is of the primer used in the analysis of a nucleotide sequence of the present raffinose synthase gene. 5-SC-2 (SEQ ID NO: 23) is used in the analysis of the present nucleotide sequence in the 3'-terminal region.

List 6

The nucleotide sequences shown in List 6 (SEQ ID NOS:

24-27) are of the primers used in the analysis of the present raffinose synthase gene of beet. The base represented by the symbol "i" is inosine. The bases shown in parentheses mean that a mixture of those bases was used in the synthesis. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 7

The nucleotide sequences shown in List 7 (SEQ ID NOS: 28-35) are of the primers synthesized on the partial nucleotide sequences of the beet raffinose synthase gene. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 8:

The nucleotide sequences shown in List 8 (SEQ ID NOS: 36 and 37) are of the primers used in the analysis of the cDNA nucleotide sequence of a raffinose synthase gene of mustard. The base represented by the symbol "i" is inosine. The bases shown in parentheses mean that a mixture of those bases. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 9:

The nucleotide sequences shown in List 9 (SEQ ID NOS: 38 and 39) are of the primers synthesized on the partial nucleotide sequences of the mustard raffinose synthase gene. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 10:

The nucleotide sequences shown in List 10 (SEQ ID NOS: 40-46) are of the primers used in the analysis of the nucleotide sequences of raffinose synthase gene of mustard and rapeseed. The symbol "RV" as used after the primer number means that the primer referred to by this symbol has an antisense sequence.

List 11:

The nucleotide sequences shown in List 11 (SEQ ID NOS: 47 and 48) are of the primers used in the amplification of 5'-terminal region of a mustard raffinose synthase gene. 11-SacI-BjN (SEQ ID NO: 47) is a primer whose SacI restriction site is added to the nucleotide sequence represented by the 4th to 29th nucleotides in SEQ ID NO: 6. 11-SacI-BjintRV (SEQ ID NO: 48) is an antisense primer having a nucleotide sequence corresponding to the nucleotide sequence represented by the 1164th to 1188th nucleotides in SEQ ID NO: 6.

List 12:

The nucleotide sequences shown in List 12 (SEQ ID NOS: 49 and 50) are of the adapters added to a mustard cDNA. These synthetic DNA take a double-stranded form when mixed together because they are complementary strands. This adapter has cohesive ends of cleavage sites for the restriction enzymes BamHI and SacI on both termini, and contains the restriction sites for the restriction enzymes BamHI and SacI in the double-stranded region.

List 13:

The nucleotide sequences shown in List 13 (SEQ ID NOS: 51-53) are of the primers used in the confirmation of inserting direction of the mustard-derived raffinose synthase gene. 13-35S-3 (SEQ ID NO: 51) is a primer of sense to 35S promoter. 13-B-2RV (SEQ ID NO: 52) is an antisense primer having the nucleotide sequence represented by the 593rd to 622nd nucleotides of SEQ ID NO: 6, 13-B-8 (SEQ ID NO: 53) is a sense primer having the nucleotide sequence represented by the 1110th to 1138th nucleotides in SEQ ID NO: 6.

As described hereinabove, according to the present invention, it is possible to provide raffinose synthase genes which can be utilized in techniques for varying expression level and activity of raffinose synthase in plants.

SEQUENCE LISTING FREE TEXT

SEQ ID NO: 9 to SEQ ID NO: 20: Designed oligonucleotide primer to obtain raffinose synthase gene.

SEQ ID NO: 21 and SEQ ID NO: 22: Designed oligonucleotide primer to obtain raffinose synthase gene, n is i, r is a or g.

SEQ ID NO: 23: Designated oligonucleotide primer to obtain raffinose synthase gene.

SEQ ID NO: 24 to SEQ ID NO: 27: Designed oligonucleotide primer to obtain raffinose synthase gene, n is i, y is t or c, r is a or g.

SEQ ID NO: 28 to SEQ ID NO: 35: Designed oligonucleotide primer to obtain raffinose synthase gene.

SEQ ID NO: 36 and SEQ ID NO: 37: Designed oligonucleotide primer to obtain raffinose synthase gene, n is i, r is a or g.

SEQ ID NO: 38 to SEQ ID NO: 48: Designed oligonucleotide primer to obtain raffinose synthase gene.

SEQ ID NO: 49 and SEQ ID NO: 50: Designed oligonucleotide linker to obtain raffinose synthase gene.

SEQ ID NO: 51 to SEQ ID NO: 53: Designed oligonucleotide primer to confirm direction of the inserted raffinose synthase gene.